VIROLOGY 216, 146–157 (1996)
ARTICLE NO. 0041

Sequence, Function, and Phylogenetic Analysis of an Ascovirus DNA Polymerase Gene

BRETT J. PELLOCK,* ALBERT LU,* RICHARD B. MEAGHER,* MICHAEL J. WEISE,† and LOIS K. MILLER*,‡,[1]

*Department of Genetics, †University Computing and Networking Services, and ‡Department of Entomology, University of Georgia, Athens, Georgia 30602-2603

Received September 22, 1995; accepted November 29, 1995

We have sequenced a 5.5-kb region of the DNA genome of the *Spodoptera* Ascovirus (SAV) containing a DNA polymerase gene. The gene codes for a 1104-amino-acid polypeptide with seven motifs characteristic of DNA polymerases and three additional motifs associated with polymerases possessing 3′ to 5′ exonuclease activity. The SAV DNA polymerase gene was able to functionally substitute for a baculovirus DNA polymerase gene in a transient assay that relies on origin-specific reporter plasmid DNA replication. Analysis of the predicted DNA polymerase sequence using neighbor-joining and protein parsimony algorithms indicated that this gene was only distantly related to other known viral and cellular DNA polymerases. The SAV DNA polymerase gene is the first ascovirus gene to be identified and sequenced. The molecular phylogenetic analyses of this gene supports the placement of insect ascoviruses in a separate virus family.

INTRODUCTION

Ascoviruses are DNA-containing insect viruses with a complex morphology and unique cytopathology which suggest that they comprise a new family of viruses (Federici, 1983). The large double-stranded DNA genomes of ascoviruses range in size from 140 to 180 kbp (Federici *et al.,* 1990). The virions vary in shape from reniform to bacilliform and consist of a structurally complex inner particle surrounded by an outer envelope with a reticulated surface (Federici, 1983; Federici *et al.,* 1990). Initial signs of ascovirus infection are nuclear hypertrophy followed by nuclear disintegration and division of the cell into 20 to 30 membrane-bound vesicles (Federici, 1983). Vesicle formation, which is highly reminiscent of apoptosis, occurs within 12 to 16 hr after infection, and virion maturation occurs during this process (Federici, 1994). The name "ascovirus" derives from the Greek word "asco" meaning "sac," since vesicle formation appears to be closely associated with the replication process.

Ascoviruses appear to be vectored on the ovipositors of parasitoid wasps (Hamm *et al.,* 1985) and the ensuing infection in the host lepidopteran larvae is characterized by stunted larval growth and development (Browning *et al.,* 1982; Carner and Hudson, 1983; Federici, 1983; Govindajaran and Federici, 1990; Federici and Govindajaran, 1990). Infected larvae experience difficulty molting, arrested development, reduced feeding, reduced weight gain, and lethargy, and have high concentrations of subcellular vesicles in their hemolymph, giving it a characteristic opaque, milky-white appearance. Ascovirus infections are chronic and ultimately fatal, with larval death often occurring 20 or more days postinfection (Federici, 1993).

The best-characterized ascoviruses are *Trichoplusia* ascovirus (TAV) (Browning *et al.,* 1983), *Spodoptera* ascovirus (SAV) (Hamm *et al.,* 1985), and *Heliothis* ascovirus (HAV) (Carner and Hudson, 1983), although virus-like particles resembling ascovirus virions have been described in other insects, including several Brazilian fire ant species (*Solenopsis* spp.) (Avery *et al.,* 1977), the clover cutworm (*Scotogramma trifolii*) (Federici, 1982), and the common looper (*Autographa precationis*) (Hamm *et al.,* 1986). SAV appears to have a limited host range, infecting only species of the genus *Spodoptera* (Lepidoptera: Noctuidae), whereas TAV and HAV have wider, overlapping host ranges that include noctuids of the genera *Spodoptera, Heliothis, Trichoplusia*, and *Autographa* (Hamm *et al.,* 1986). TAV and HAV also have a broader tissue tropism, infecting the tracheal matrix, epidermal cells, and, to a lesser extent, fat body cells, whereas SAV infection is primarily limited to fat body tissue (Federici and Govindajaran, 1990). Restriction endonuclease and Southern hybridization analyses of ascovirus DNA indicate a close relationship between the ca. 180-kbp genomes of HAV and TAV. SAV is a more distinct species and has a smaller genome size of ca. 140 kbp (Federici *et al.,* 1990).

In this study we report the identification and characterization of the first ascovirus gene. We present the nucleotide sequence of a 5.5-kb *Sma*I fragment of the SAV genome with an open reading frame (ORF) that codes

Sequence data from this article for SAV DNA polymerase gene have been deposited with the EMBL/GenBank Data Libraries under Accession No. U35732.

[1] To whom correspondence and reprint requests should be addressed at Department of Entomology, University of Georgia, Athens, GA 30602–2603. Fax: (706) 542–2279. E-mail: Miller@bscr.uga.edu.

0042-6822/96 $12.00

for a large polypeptide with sequence motifs characteristic of DNA polymerases (Spicer *et al.,* 1988; Wong *et al.,* 1988). We also provide genetic evidence that this ORF encodes a DNA polymerase. Comparing this sequence with sequences of other known DNA polymerases, we have constructed a series of unrooted taxonomic trees that place the SAV DNA polymerase in a separate position on the trees. This phylogenetic analysis thus supports the placement of ascoviruses in a new virus family.

MATERIALS AND METHODS

Virus, larval inoculation, and virus vesicle preparation

The SAV isolate was originally provided by Dr. Brian Federici (University of California, Riverside). The virus was propagated by inoculating early to middle fourth-instar *Spodoptera frugiperda* larvae using a small pin dipped in a SAV vesicle suspension. Inoculated larvae were incubated at 27° for 9 days. The hemolymph of infected larvae was collected into an Eppendorf tube on ice. Viral vesicles were pelleted in a microcentrifuge (14,000 rpm for 30 sec), resuspended in an equal volume of 0.1 *M* sodium phosphate, pH 7.4, frozen in liquid nitrogen, and stored frozen at −80° until further use.

Purification of virions and isolation of SAV genomic DNA

Virion purification and DNA preparation was performed using the protocol of Federici *et al.* (1990) with several modifications. SAV vesicles obtained from 500 μl to 1 ml of hemolymph were thawed on ice, pelleted at 2000 *g* for 5 min, and resuspended in 5 ml of 1% Triton X-100. Vesicles were disrupted by vigorous pipetting for 5 min followed by vigorous vortexing for 2 min. The disrupted vesicles were centrifuged at 2000 *g* for 5 min and the resulting supernatant layered onto a 20–60% (w/w in PBS, pH 6.2) sucrose gradient. To maximize yields, the pellet was resuspended in an additional 5 ml of 1% Triton X-100, sonicated at power 8 with a Branson Sonifier 450 (Branson Sonic Power Company, Danbury, CT) for 15 sec at 90% duty cycle, and the suspension was centrifuged at 2000 *g* for 5 min. The supernatant was layered onto another 20–60% (w/w in PBS) sucrose gradient and both gradients were centrifuged at 72,100 *g* in a Beckman SW 28.1 rotor for 2 hr at 12°. Two bands were observed in these gradients, one at ca. 30% sucrose and one at ca. 55% sucrose; both bands were collected from the tubes, combined, and diluted with 5 vol of PBS. The viruses were pelleted by centrifugation through a sucrose cushion (25% sucrose, 5 m*M* NaCl, 10 m*M* EDTA) at 110,000 *g* in a Beckman SW 28.1 rotor for 75 min at 12°. The pelleted virions were resuspended in 200 μl of disruption buffer (10 m*M* Tris–HCl, pH 7.6, 10 m*M* EDTA, 0.25% SDS), 10 μl of a proteinase K (Sigma Chemical Co., St. Louis, MO) stock solution (20 mg/ml) was added, and the suspension was incubated overnight at 37° with gentle agitation. SAV DNA was extracted twice with buffer-saturated phenol, extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), extracted once with chloroform:isoamyl alcohol (24:1), precipitated with 0.1 vol 3.0 *M* sodium acetate, pH 5.2, and 2 vol of absolute ethanol, pelleted using a microcentrifuge,

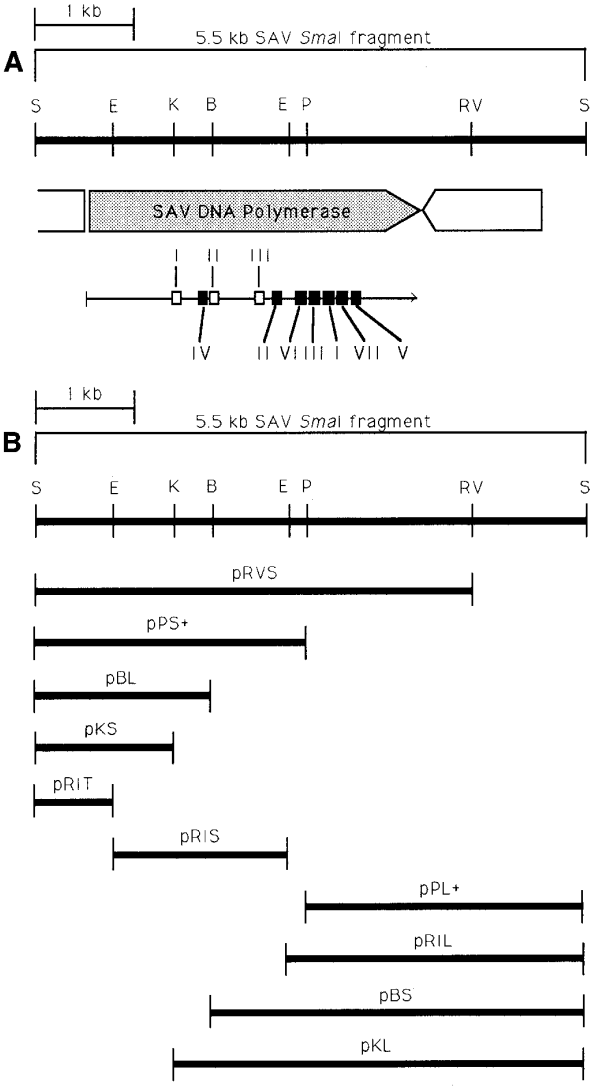

FIG. 1. Partial restriction maps and subclones of the insert within pDP, the plasmid containing the SAV DNA polymerase gene. (A) Restriction map of the 5.5-kb *Sma*I fragment of the SAV genome, the location of the ORF coding for SAV DNA polymerase (shaded arrow) and the positions of flanking ORFs. The linear order and approximate spatial locations of conserved motifs of DNA polymerase-associated exonuclease domains I through III (open squares and numerals above the line arrow) and conserved DNA polymerase regions I through VII (filled squares and numerals below the line arrow) are indicated. The insert in pSAVHE6 encompassed conserved region IV; the sequence of this insert is presented in Fig. 2 and the conserved motifs are compared in Fig. 3. Key for restriction endonuclease sites: S, *Sma*I; E, *Eco*RI; K, *Kpn*I; B, *Bam*HI; P, *Pst*I; RV, *Eco*RV. (B) Schematic maps of pDP subclones. The subclones contain, within the pBSKS+ vector, the portions of the pDP insert under which they are aligned. Plasmid pPL− contains the same insert as pPL+ in the opposite orientation.

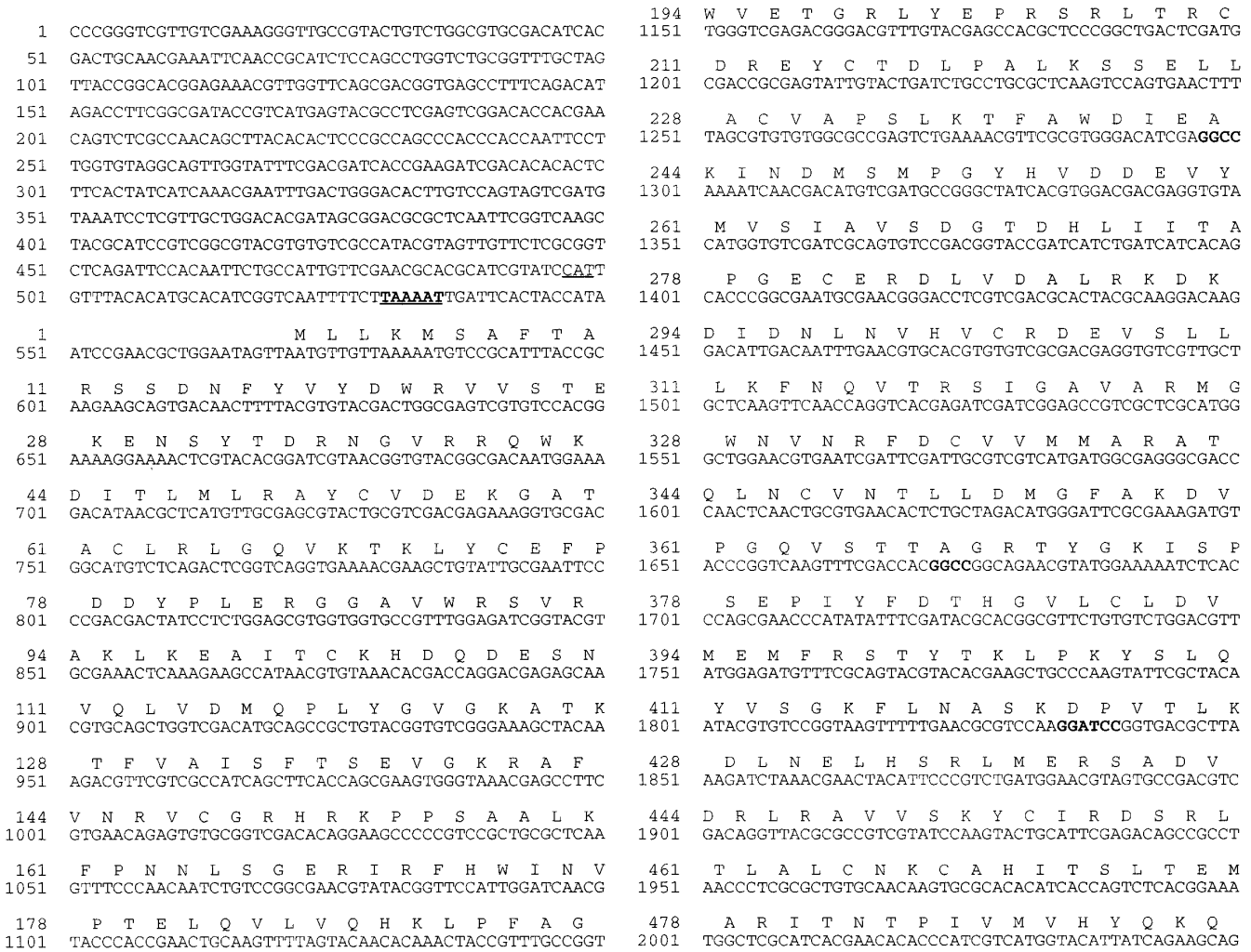

FIG. 2. DNA sequence of the 5555 nucleotide *Sma*I fragment from SAV. The predicted protein sequence of the DNA polymerase encoded by the 3.3-kb ORF is indicated above the DNA sequence in single-letter amino acid code. The numbers along the left indicate the number of the left-most nucleotide or amino acid in the line. The *Hae*III sites that flank the 375-nucleotide fragment contained in pSAVHE6 and the *Bam*HI site used to frameshift the DNA polymerase gene are indicated in boldface type. A possible TATA motif and a polyadenylation signal (shared with the termination codon) are underlined and in boldface type. The start and stop codons (CAT and TCA, respectively) of a bottom-strand ORF (downstream of the DNA polymerase gene) and the start codon (CAT) of a bottom-strand ORF that does not terminate prior to the 5′ terminus of the sequenced region (upstream of the DNA polymerase gene) are underlined.

washed with 70% ethanol, and resuspended in Tris–EDTA buffer, pH 8.0.

Cloning and sequence analysis of SAV *Hae*III fragments

SAV genomic DNA was digested to completion using *Hae*III (GIBCO BRL, Gaithersburg, MD) and the fragments were randomly cloned into the *Sma*I site of pBSKS+ (Stratagene, La Jolla, CA). Plasmids with an insert size of 300 to 500 bp were chosen for DNA sequence analysis; pSAVHE6 was one of these plasmids. The termini of the SAV DNA fragments were sequenced using T3 and T7 universal primers. The predicted sequences of open reading frames found in the sequences were analyzed using both Blast (Altschul *et al.,* 1990) and Fast A (Pearson and Lipman, 1988) searches of the Swiss-prot and GenPept databases.

Identification and cloning of the 5.5 kb SAV SmaI fragment

SAV genomic DNA was digested with a wide variety of restriction endonucleases and the resulting fragments were separated by electrophoresis on 0.7% agarose gels, Southern blotted, hybridized to nick-translated plasmid pSAVHE6 DNA, and exposed to autoradiography film. A 5.5-kb SAV *Sma*I fragment hybridized to pSAVHE6; the equivalent 5.5-kb *Psp*AI (*Sma*I isoschizomer) fragment was gel-isolated from a *Psp*AI digest of SAV DNA and ligated into *Psp*AI-digested pBSKS+. Identity of the fragment within the resulting plasmid, pDP, was confirmed by Southern blot analysis.

```
 494  Q R R M F H L M F S E C M R E N V
2051  CAACGCAGAATGTTCCATCTCATGTTCTCCGAGTGCATGCGAGAGAACGT

 511  A M Q D D F G L A N A A P F E T V
2101  CGCGATGCAAGACGACTTCGGACTCGCCAACGCCGCGCCCTTCGAAACCG

 528  A A T Q K K Q L S Y S G A Y V K
2151  TAGCCGCGACACAAAAGAAGCAGCTCAGCTACAGCGGGGCTTATGTCAAA

 544  D P E P G L Y S M V G S L D V N N
2201  GACCCAGAACCGGGACTGTATAGCATGGTGGGATCGTTGGACGTGAACAA

 561  M Y P T L M I A Y N L C Y S T V V
2251  TATGTATCCCACGCTTATGATCGCGTACAATCTTTGCTACAGCACCGTAG

 578  D E H S P S A F T D D H F E Y I
2301  TCGACGAACACTCACCGTCGGCTTTCACGGACGATCACTTTGAATACATT

 594  R W E D H V G C E H D P V Q V E L
2351  CGATGGGAGGACCACGTCGGTTGCGAACACGATCCCGTACAAGTGGAACT

 611  K S L R S M F E M K A L T D K R K
2401  CAAAAGTCTGAGGAGCATGTTCGAAATGAAAGCGTTGACCGACAAACGCA

 628  R I G V T A I T K Y F K P I Q G
2451  AACGGATCGGTGTCACCGCAATCACAAAGTATTTTAAGCCCATTCAAGGC

 644  P R I I Q K I M D D R Y A E S T C
2501  CCGAGGATCATTCAAAAAATAATGGACGATCGTTACGCGGAATCGACGTG

 661  R Y D D G D E F C E N D D E K E V
2551  TCGCTATGACGACGGCGATGAATTCTGCGAAAACGATGACGAGAAGGAGG

 678  I A A I K T P L E M Q R A A I R
2601  TGATCGCCGCCATTAAAACTCCCCTCGAAATGCAAAGGGCCGCGATACGT

 694  L K V L K S R A S G G S V L C A K
2651  TTGAAAGTGTTAAAGTCTCGAGCATCTGGCGGATCGGTGTTGTGCGCCAA

 711  Q C V K I L K T R R G I L P D L V
2701  ACAATGTGTGAAAATATTGAAAACGAGGCGTGGCATTCTACCCGATTTGG

 728  E C L L Q A R K R V R G N M K S
2751  TCGAGTGTCTTCTGCAGGCGAGGAAACGCGTGCGCGGTAACATGAAATCC

 744  V T D P M A R D I L D K S Q L A Y
2801  GTGACCGATCCGATGGCGCGGGACATTCTGGACAAGAGCCAATTGGCGTA

 761  K V T A N S I Y G S T G A S N G K
2851  CAAGGTGACGGCGAACTCTATATACGGATCGACGGGTGCCTCGAACGGTA

 778  L P C Q N V A K V T T A L G R M
2901  AACTACCGTGTCAGAACGTTGCCAAGGTGACCACGGCGCTGGGTAGGATG

 794  T I L Q A I E I A R T E R N V T T
2951  ACCATACTGCAAGCCATCGAAATTGCACGAACCGAGCGTAACGTCACAAC

 811  I Y S D T D S M Y V Q L Q D D P A
3001  GATATACAGCGATACCGACTCGATGTACGTGCAACTACAGGACGATCCCG

 828  S N Q D P W T Y V R E L A T H I
3051  CGTCAAACCAAGATCCATGGACGTACGTGCGCGAACTGGCCACACACATC

 844  T S K L R E P M V I E A E D D I H
3101  ACGTCTAAACTACGCGAGCCGATGGTGATCGAGGCCGAAGACGACATTCA

 861  A K V L F L G K K C Y I G R K L F
3151  CGCCAAAGTGCTGTTCCTCGGCAAGAAATGCTACATCGGACGCAAACTGT

 878  R D G S V A R D L D W H V V I T
3201  TCCGGGACGGTAGCGTGGCTCGCGACCTGGACTGGCACGTTGTGATCACG

 894  V R R D H S E Y V K S A Y K P P V
3251  GTGCGTCGGGACCACAGTGAGTATGTGAAGAGTGCGTACAAGCCGCCCGT

 911  Y K V F A D C T H D Q F V T S I A
3301  GTACAAAGTGTTCGCGGACTGTACGCACGACCAGTTCGTCACATCGATTG

 928  E S C L R L M R R T V P C E M L
3351  CGGAATCGTGTCTGAGGTTGATGCGACGCACGGTTCCATGTGAGATGCTC

 944  T K T S E V R N L G D G C Q L V L
3401  ACGAAGACGAGCGAAGTGCGTAACCTGGGTGACGGTTGTCAGTTGGTACT

 961  C K K T M S W M W G D Y K V A R H
3451  GTGCAAAAAGACCATGTCCTGGATGTGGGGCGATTATAAAGTGGCCAGAC

 978  E Q H D R L Q R E N P K W L R D
3501  ACGAACAACACGATCGATTACAGCGCGAAAACCCCAAGTGGCTGCGAGAC

 994  Y Y V S K L P A P A R L S I L L T
3551  TATTACGTTTCGAAACTACCGGCTCCGGCTAGATTGAGCATCCTGCTCAC

1011  D R A R P P V E G G R I E F L N V
3601  GGACAGAGCCCGACCACCGGTCGAAGGTGGCCGAATCGAGTTTCTAAACG

1028  K T E T K S A L A I E E I S Y F
3651  TGAAAACCGAAACAAAGTCGGCGCTCGCCATCGAAGAAATATCCTATTTC

1044  K D N P G C A Q V D N M Y Y I Q Q
3701  AAGGACAATCCCGGCTGTGCACAAGTCGACAACATGTACTACATACAACA

1061  L V N P L T K V S E A V W R R S N
3751  GCTGGTCAACCCTCTCACTAAGGTTTCCGAAGCGGTGTGGCGCCGCAGTA
```

FIG. 2—*Continued*

DNA sequencing and plasmid construction

Exonuclease III (GIBCO BRL) was used to generate nested sets of deletion clones of pDP subclones (Fig. 1) using methods previously described (Henikoff, 1987). Dideoxynucleotide chain termination DNA sequencing was performed using the USB Sequenase kit (United States Biochemical Corp., Cleveland, OH). Additional sequence data were obtained using an Applied Biosystems automated sequencer at the University of Georgia Molecular Genetics Instrumentation Facility. Both strands of the entire 5.5-kb insert of pDP were sequenced. Plasmids pPL+ and pPL−, subclones of pDP that contain the same SAV sequences in the opposite orientation in the vector, facilitated sequencing. pPL− was derived from pPL+ as follows: pPL+ was digested to completion with *Sma*I (GIBCO BRL), which cuts within SAV sequences, and *Eco*RI (GIBCO BRL), which cuts within the multiple cloning site of pBSKS+. Both the vector and insert were gel-purified, treated with the Klenow fragment of *Escherichia coli* DNA polymerase I (GIBCO BRL) and dNTPs, and religated to one another. This cloning strategy preserved certain restriction endonuclease sites within the vector, which allowed pPL− exonuclease deletion clones to be constructed using the identical strategy as was used for pPL+.

The plasmid pDPfs, which has a frameshift mutation in the SAV DNA polymerase gene, was generated as follows: pBSKS+ was digested to completion with *Bam*HI (GIBCO BRL) and gel-purified. The linearized plasmid was treated with Klenow and dNTPs and religated to produce pBSKS+ΔHI, which lacks the *Bam*HI site in its muliple cloning site. pBSKS+ΔHI and pDP were digested with *Psp*AI. The 5.5-kb SAV fragment and pBSKS+ΔHI were gel-purified and ligated to produce pDPΔHI, a plasmid with a unique *Bam*HI site in the SAV DNA polymerase gene. pDPΔHI was digested to completion with *Bam*HI, gel-purified, treated with Klenow and dNTPs, and religated to produce pDPfs. This frameshift mutation, predicted to cause premature termination of protein translation five amino acids

```
1078    S  V  N  I  A  I  A  P  F  I  Y  Y  N  K  V  L
3801  ACAGCGTTAACATTGCCATCGCGCCGTTCATCTATTACAATAAGGTTTTA

1094  A  Q  L  S  T  R  F  K  I  S  Q  *
3851  GCTCAATTGTCAACTAGGTTTAAGATTTCGCAATAAAACTACAATGGGTA
3901  CAAAACAAAATTTGTTTTATTTCATTTTCTCAAATACACGATTCGATCCA
3951  CCAGGGTTTCCTCGGTATATTCCTAGAAGTCCACATGCGATCCGCACATG
4001  ATGTAGGTCGCATCTCGTCGAACCGTTGCGCCGACAATCTATTCATCTGT
4051  ACGTGCATCAATGTGATGAAGTCATCGAGACACTGTGCAAAATCAACGGG
4101  GCAGCTGCCGACGATGCGGTCCGTCAACGTGCCGACAATCGTGCACTTGT
4151  GACGCCTCAAGTCATCGACGTAGTGTCTAACGGTAGCGGCCAGCAGTTCA
4201  CGATCTCGTCCGAGTGTGGGCGTGTTGAATACCGTGGTCGGTCTCAGCGC
4251  GTACACGAGCGGTGTGATACGACGATTGTCGTTCCTGATCGTGCCGTTCA
4301  GGATTCTCGAGTGAGTCTTGTGTATTTCATGGTAGTTACGTAGATATGCA
4351  CCCTCGGTGGGATCGATGACGCTAGTCAAACGCATGTGTGGATGGGTAGA
4401  GAACACGGCCACCGAATGGTGATCACGCACACCGCCTATGGTTCGCATTA
4451  TTCCGTAAGTGCCGGGTACTCGTGCCAATATCAACTCGGCACACACCCAC
4501  ACCGACAGATGTTTCGAAGTCGCTACTGCGCAAGCCTCTGCTAGCGCAGC
4551  GTGTAAAATAGTTATGGAATTGGAATGTAATGCGTTCACGGGCCGCTGTT
4601  GCTCGATCAAGTCGATCAGCTCGTCCACCAGTTGCAGTCGATATCTTCTC
4651  GGGTCCGATGTCTGTAGCCAAGTCGATGAAATCGTGCGTGGTACGTGCGG
4701  AAATGCTTCCCCACTCCAATCTATGGTGCCGTATTCGAAAACACACCTAC
4751  ACTTGCCGATACACGATCGAAACGTATCGGATCTGGTGGTATTCATCTCG
4801  TGGCCAACACACCGAACAGTACCAACAATCCGATTATCGCCAACGTGACG
4851  TAGGTAGACGACGGTGACTTGGACCTCTCGACTGCTACTTCCTCTCCTCC
4901  GCCCAGCTCCGTATTGTAGTCGTCCAGCTGCTTTTGCCAATCGACGTAGT
4951  TACTCGAGAACTGTGCCAGTGCGATGGGGTTCTGTAGAATCTTTTTCATG
5001  GACACTTGAATAATTAACGCGAGCCCAACGCAGCCGCTGCACTGGTCCAA
5051  CGTCAGAAAGCCACTGCTGGCTCTCTTTAAGAATACAACGGGCTTAGGCA
5101  CCGTCCTGTTGTATGCGTCGATTGTGGCTTTGGAGGATAATTCACCTTCG
5151  ATGCCAAACACTGAAGTGTACATACTCAATGCGGCCGGCTTTATTTGTAG
5201  ATAGCAACTTGGCCGTCAACGATAACCGTACGCAGATGCCGTTCCGGAAC
5251  GTGCTCAGAGCTCGTTTCTCGTAGATGGTGACACTGTATTATACTCGGGT
5301  GGAATTATGATTGTTGGAGCTTCCGCCACCGATGATGACAACTTTAATGT
5351  ACCCAATAAACCCGATAAACTTACCGGTTGTACGACTGTAACATAAGCGT
5401  AACTCATAATTTAATTGGTTTTATTGATCACGTATCGATTTATCATCAAA

5451  GTGGGAGCAGTCGTTTCATCCAAGAATGTATGCGAACAACAAACAAGCAC
5501  ACACGATCCAAACCAATTTCCTATTATCCACATAGATTTGTGACAGCTTC
5551  CCGG
```

FIG. 2—*Continued*

downstream of the frameshift, was confirmed by DNA sequence analysis.

Transient expression and CAT assays

Transfections were performed in the IPLB-SF-21 cell line (SF-21) (Vaughn *et al.,* 1977) using lipofectin (GIBCO BRL) and the reporter plasmid pCAPCAT (Thiem and Miller, 1990), as previously described (O'Reilly *et al.,* 1992). Plasmids comprising the *lef* library used in this study were described by Lu and Miller (1995) except that pPstI-K (Todd *et al.,* 1995), containing both *39K* and *lef-11,* was used in place of pNspAfI (which contains *39K*) and pH3R (which contains *lef-11*). Chloramphenicol acetyltransferase (CAT) assays were performed as described (Gorman *et al.,* 1982; Passarelli and Miller, 1993). The results of three separate CAT assays were quantitated using a Molecular Dynamics Phosphorimager and the standard deviations of the data points were determined.

Sequence alignments and construction of unrooted phylogenetic trees

The Pileup program from the Wisconsin package (version 8.0, Genetics Computer Group, 1994) was used to generate multiple sequence alignments. Highlighted comparisons of these were produced using the Boxshade program, version 2.7, contributed to the public domain by author Kay Hofmann; email:khofmann@isrecsun1.unil.ch). Phylogenetic trees were produced using programs (protpars, neighbor, seqboot, consense) from the Phylip package (Felsenstein, 1993). Trees were generated using both protein parsimony and neighbor-joining algorithms. Bootstrap analysis was performed on all sets of data, and consensus trees were obtained. Analyses were performed on alignments from complete DNA polymerase protein sequences and from peptide sequences representing the conserved sequence motifs of the DNA polymerase molecules.

RESULTS

Identification, cloning, and sequencing of the SAV DNA polymerase gene

SAV genomic DNA was digested with *Hae*III, and the resulting *Hae*III fragments were cloned and 31 were sequenced. The peptide sequences of hypothetical SAV open reading frames were compared with other proteins in available sequence databases. The peptide sequence encoded by the insert within one sequenced plasmid, pSAVHE6, showed significant homology to the conserved residues in sequence motif IV of DNA polymerase molecules (Figs. 1A, 2, and 3) using Fast A and Blast search programs. The pSAVHE6 DNA hybridized to a 5.5-kb *Sma*I fragment of the SAV genome in Southern blot analysis.

This 5.5-kb fragment was cloned as plasmid pDP and both strands were sequenced using a variety of subclones (Fig. 1); nested sets of exonuclease deletion clones derived from pPS+, pPL+, and pPL− (Fig. 1B); and custom-synthesized oligonucleotides. The sequence of the 5.5-kb *Sma*I fragment (Fig. 2) revealed a 3.3-kb ORF that codes for a 1104-residue polypeptide. The 3.3-kb ORF was flanked by sequences containing smaller ORFs (Fig. 2) with no obvious homologs in available DNA sequence databases.

The predicted 1104-residue polypeptide contains seven sequences which are similar to and have the same linear order as the seven conserved sequence motifs (IV, II, VI, III, I, VII, and V) of many DNA polymerases (Figs. 1A and 3). In addition, this protein sequence contains three motifs that align with three conserved regions implicated in DNA polymerase 3′–5′ exonuclease activity (Fig. 4).

The SAV DNA polymerase gene substitutes for the AcMNPV DNA polymerase gene in a DNA replication-dependent transient expression assay

To determine whether the cloned SAV DNA polymerase gene encoded a functional DNA polymerase, we took

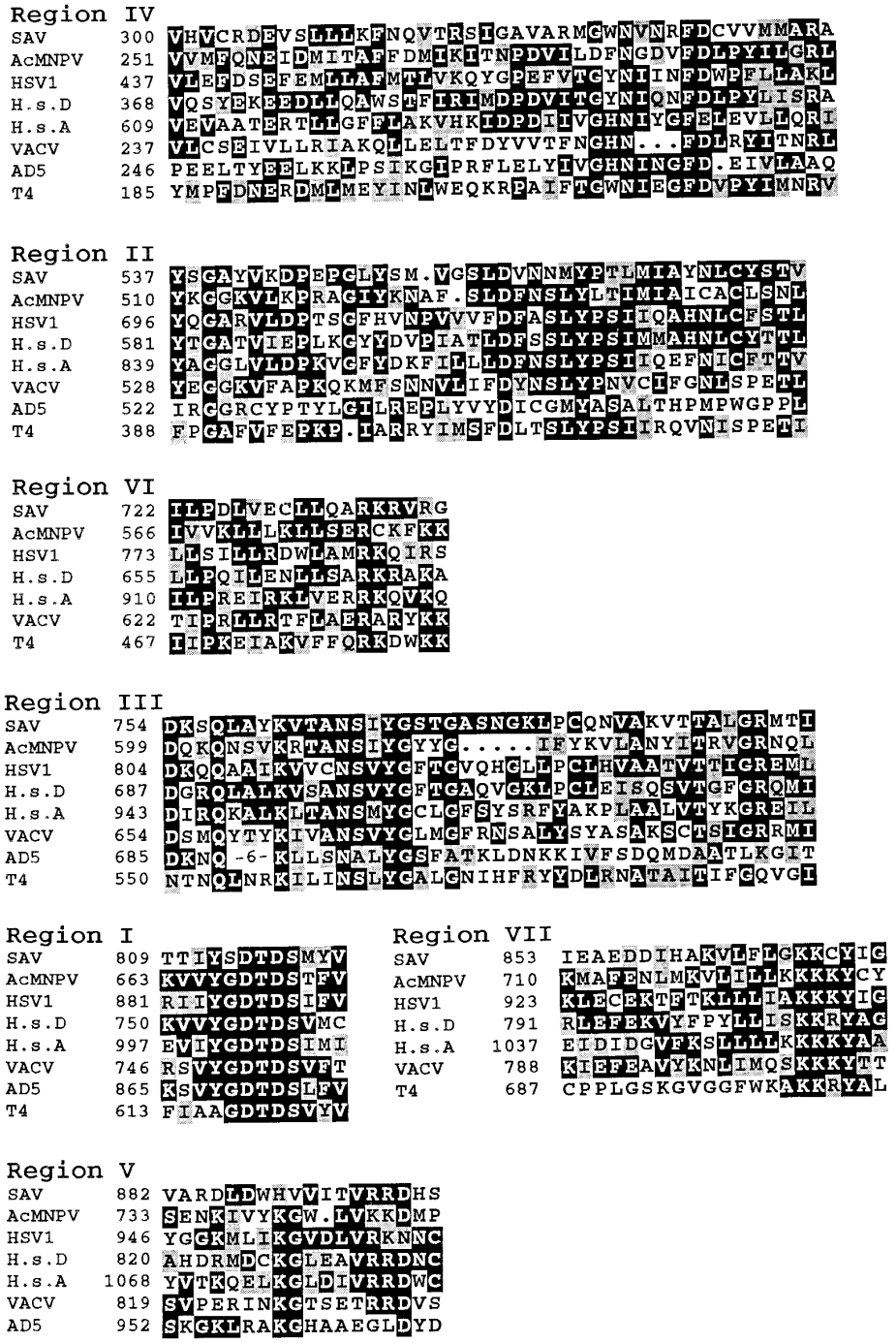

FIG. 3. Alignments of the seven conserved motifs of SAV DNA polymerase and several other DNA polymerases. The motifs are organized in the conserved linear order: IV, II, VI, III, I, VII, V. Identical residues are shaded black; similar residues are shaded gray. The numbers to the left of each SAV sequence correspond to the amino acid numbers of the SAV DNA polymerase sequence shown in Fig. 2. Key: SAV, *Spodoptera* ascovirus; AcMNPV, *Autographa californica* nuclear polyhedrosis virus; AD5, human adenovirus type 5; T4, *E. coli* phage T4; HSV1, herpes simplex virus type 1; VACV, vaccinia virus; H.s.D, human DNA polymerase δ; H.s.A, human DNA polymerase α. The sequences of those DNA polymerases that lack certain motifs are naturally omitted from the appropriate box-shaded alignments.

advantage of a DNA replication-dependent, transient expression assay system which was originally developed to define the genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) involved in late gene expression (Passarelli and Miller, 1993). Baculovirus late gene expression depends on DNA replication and this dependence is also observed in transient expression assays (Lu and Miller, 1995) that use a late viral gene promoter in a reporter plasmid, pCAPCAT (Thiem and Miller, 1990), which also carries an AcMNPV "hr"

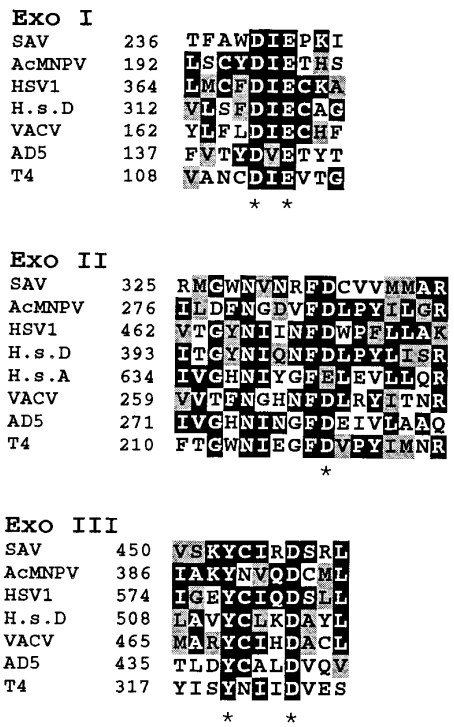

FIG. 4. Alignments of conserved sequence motifs involved in DNA polymerase 3′–5′ exonuclease activity. Exonuclease motifs I, II, and III are shown for SAV DNA polymerase and others as indicated in the legend for Fig. 3. Asterisks indicate the five residues thought to comprise the 3′–5′ exonuclease active site (Bernad *et al.,* 1989; Morrison *et al.,* 1991).

sequence . The hr sequence is required for reporter plasmid DNA replication in this expression system (Lu and Miller, 1995) and is thought to serve as an origin of DNA replication for both the plasmid and the virus (Pearson *et al.,* 1992). In SF-21 cells, 18 viral genes, 9 of which are involved in the replication or stability of the reporter plasmid (Lu and Miller, 1995), are necessary and sufficient to achieve substantial transient expression from the late AcMNPV promoter when cotransfected with the pCAPCAT reporter plasmid (Todd *et al.,* 1995).

The DNA polymerase gene of AcMNPV is one of the 9 replication-specific genes (Lu and Miller, 1995) and one of the 18 genes required to optimize expression from the reporter plasmid pCAPCAT in transient expression assays. The set of plasmids comprising the 18 late expression factor genes (*lef*s) which collectively support transient expression from this reporter plasmid is referred to as the *lef* library. When the AcMNPV DNA polymerase clone, pDNAp (Tomalski *et al.,* 1988), is removed from the *lef* library, CAT expression drops dramatically (Lu and Miller, 1994; and Fig. 5, lanes 2 and 3).

To determine if the SAV DNA polymerase gene could substitute for the AcMNPV DNA polymerase gene in this assay, we removed pDNAp containing the AcMNPV DNA polymerase gene from the AcMNPV *lef* library and substituted equimolar quantities of pDP, the plasmid containing the SAV DNA polymerase gene (Fig. 5, lane 4). Cotransfection of pDP along with the other members of the AcMNPV *lef* library restored the level of CAT expression from pCAPCAT to approximately half the level achieved with pDNAp (Fig. 5, compare lanes 2 and 4). A plasmid, pDPfs, with a frameshift mutation at the *Bam*HI site in the SAV DNA polymerase gene (Figs. 1A and 2) was unable to substitute for pDNAp in transactivating CAT gene expression (Fig. 5, lane 5). CAT activity could be completely restored by substituting higher quantities of pDP for pDNAp (data not shown). This provides strong genetic evidence that the SAV DNA polymerase gene product can functionally substitute for the AcMNPV DNA polymerase in this assay system.

Phylogenetic analysis of the SAV DNA polymerase gene

To more thoroughly analyze the relationship of the SAV DNA polymerase gene with other know DNA polymerase genes, we compared the sequence of SAV DNA polymer-

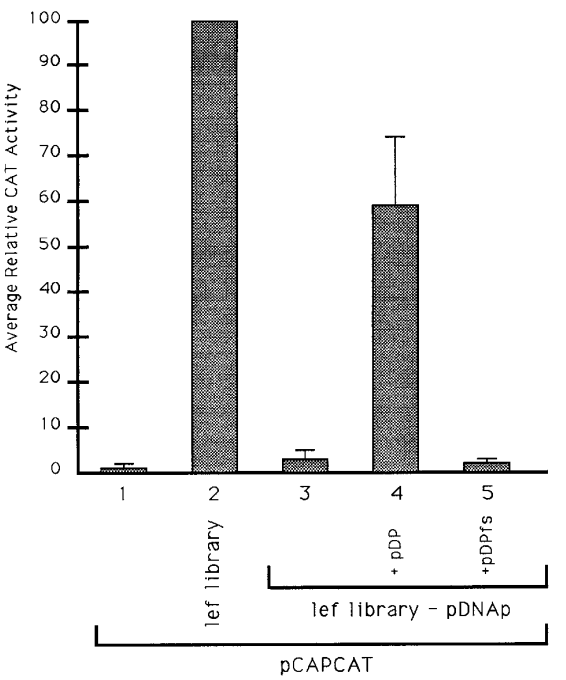

FIG. 5. The SAV DNA polymerase gene substitutes for the AcMNPV DNA polymerase gene in a DNA replication-dependent transient expression assay. Cells were transfected with the reporter plasmid pCAPCAT (lanes 1–5) and the *lef* library consisting of a set of plasmids comprising the 18 genes required to transactivate the late promoter of the reporter CAT gene (lane 2) or the *lef* library lacking the plasmid supplying the AcMNPV DNA polymerase gene (lanes 3–5). Removal of the plasmid containing the AcMNPV DNA polymerase gene was compensated by the addition of equimolar quantities of pDP (lane 4) or pDPfs (lane 5) as indicated below each bar. The bar graph indicates the relative CAT activity, the percentage conversion of chloramphenicol substrate to acetylated product compared to the pCAPCAT plus the *lef* library positive control reaction averaged over three separate iterations of the assays.

ase with 37 additional DNA polymerases representative of all known families of large DNA containing viruses (herpesviruses, poxviruses, adenoviruses, and baculoviruses), selected eukaryotes (alpha and delta), prokaryotes (eubacteria and archaebacteria), bacteriophages (with both gram-negative and gram-positive hosts), mitochondrial and extrachromosomal DNA polymerases (such as the yeast killer plasmids), and two DNA polymerases from viruses thought to be in their own classes (African swine fever virus and chlorella virus). Sequence comparison was performed by constructing a series of phylogenetic trees using three protein sequence data sets: (1) the complete DNA polymerase amino acid sequences, (2) the sequences of conserved sequence motifs I, II, and III of all the DNA polymerases analyzed, and (3) the sequences of conserved motifs IV, V, VI, and VII from the DNA polymerases containing these motifs.

Protein parsimony and neighbor joining algorithm analyses of these three different sets of DNA polymerase sequences indicated that SAV DNA polymerase does not belong to any of the established classes of DNA polymerases listed above. The six trees generated by these analyses all placed the SAV DNA polymerase on its own branch, well-distanced from other polymerases. Analysis of the entire polypeptide sequence of each of the polymerases by the neighbor-joining algorithm, for example, placed the SAV DNA polymerase in its own branch off the base of the delta DNA polymerase class (Figs. 6A and 6C). The chorella virus (CHV) DNA polymerase was also on a separate branch, as were the members of the herpesviridae (e.g., HSV, HCMV, and EBV). The general features of the tree were maintained in the six trees generated using the two algorithms to process the three data sets. The neighbor-joining tree presented in Fig.6 was the strongest of the six trees generated based on Bootstrap analysis.

Bootstrap analysis was used to analyze the integrity of each of the six trees. The bootstrap analysis of the tree shown in Figs. 6A and 6C is presented in Fig. 6B. The Bootstrap program generates 100 altered sequence data sets from each original data set and then builds 100 trees from these altered data sets; this helps to establish that enough samples have been examined to produce a biologically meaningful tree. The consensus tree generated from these 100 trees is presented in Fig. 6 along with branch percentages which reflect how often the branch was located in a given position. A high branch percentage indicates that the branch was consistently constructed, even with random changes in the data set, whereas a low branch percentage indicates that the branch was frequently constructed elsewhere in the tree. Bootstrap values of $>$70% are considered biologically significant. Generally, the Bootstrap analysis indicates that the branch integrity of this tree is good (Fig. 6B), especially within the narrowly defined classes of DNA polymerases (α polymerases, δ polymerases, herpesviruses, adenoviruses, poxviruses, baculoviruses, bacteriophages, eubacteria, archaebacteria, mitochondrial, extrachromosomal, and plasmid-derived DNA polymerases) which usually had branch integrities around 100%. Branch integrity was substantially lower for the branches between different DNA polymerase classes than within the defined DNA polymerase classes. For example, the branch integrity of DNA polymerase II of *E. coli* (E.co.II), which is known to be a novel DNA polymerase, was low, whereas the branch integrity among the alpha class of eukaryotic polymerases was strong even though they represented a range of organisms from yeast (S.c.A and S.p.A) to humans (H.s.A) and flies (D.m.A). Bootstrap analysis of the five remaining trees showed that the integrity of these trees was also good, especially among the defined classes of DNA polymerases.

The general tree structure presented in Fig. 6C places known members of a given DNA polymerase family in groups with the other members of the same family. These placements were consistently maintained in all six trees constructed. For example, the adenovirus polymerases cluster in a single branch, as do the members of the herpesviridae (e.g., HSV1, HCMV, and EBV). Although there was some variability in the position and length of the SAV DNA polymerase branch, it always behaved as a separate DNA polymerase class and was never grouped as a member of an established DNA polymerase class.

DISCUSSION

The sequence of a 5.5-kb *Sma*I fragment from *Spodoptera* ascovirus contains the complete 3.3-kb open reading frame encoding a 1104-amino acid polypeptide with sequence homology to DNA polymerases. This polypeptide contains the seven defined conserved DNA polymerase sequence motifs as well as the three domains associated with 3′–5′ exonuclease activity. The only overlap between these 10 motifs is the Exo II motif which overlaps with the carboxy terminus of polymerase motif IV. The order of the motifs is the same as that found in other known DNA polymerases. The presence and order of these 10 motifs strongly indicates that the polypeptide product of this gene is a DNA polymerase.

The SAV DNA polymerase gene can functionally substitute for a baculovirus DNA polymerase gene in a DNA replication-dependent transient expression system. The observation that an ascovirus DNA polymerase can functionally substitute for a baculovirus DNA polymerase in this assay, however, should not be construed as an evolutionary relationship between these two types of viruses. The host (*Spodoptera frugiperda*) cellular DNA polymerase appears to be able to function in the absence of the AcMNPV DNA polymerase in this assay, but transactivation is delayed and suboptimal, probably due to its low copy number (Lu and Miller, 1995). Since SAV and

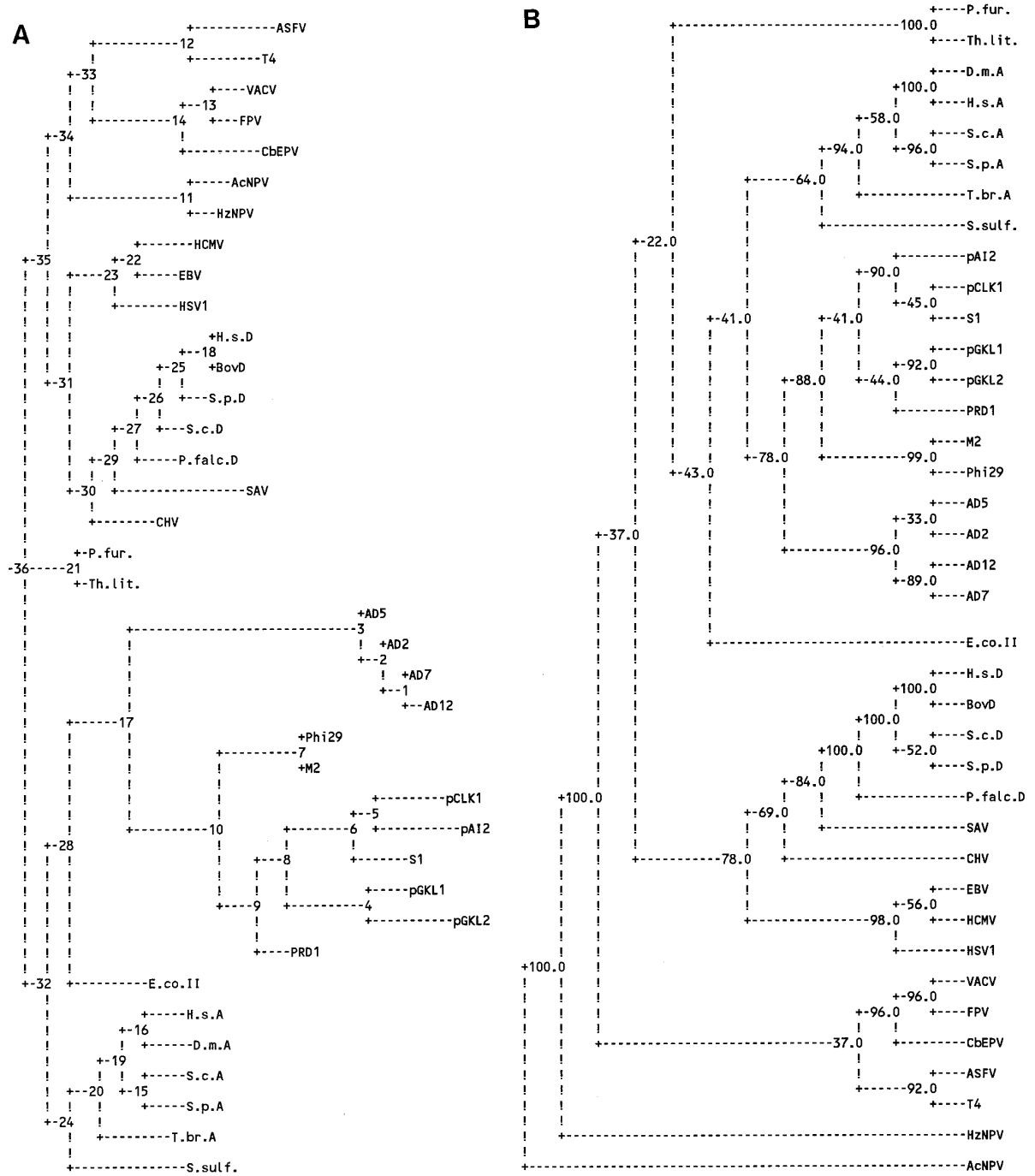

FIG. 6. Phylogenetic analysis of 38 DNA polymerases. (A) A phylogenetic tree established using a neighbor-joining algorithm and the entire sequence of each of the 38 DNA polymerases. (B) Bootstrap analysis of the tree presented in A. The numbers at the branches indicate the relative strength of the branch position. (C) A schematic presentation of the tree presented in A. Key: P.fur., *Pyrococcus furiosus* archaebacterium (Mathur *et al.,* 1991); Th.lit., *Thermococcus littoralis* archaebacterium (Perler *et al.,* 1992); S.sulf, *Sulfolobus sulfataricus* archaebacterium (Pissani *et al.,* 1992); D.m.A, *Drosophila melanogaster* DNA polymerase α (Hirose *et al.,* 1991); H.s.A, *Homo sapiens* DNA polymerase α (Wong *et al.,* 1988); S.c.A, *Saccharomyces cerevisiae* DNA polymerase I (Pizzagalli *et al.,* 1988); S.p.A, *Schizosaccharomyces pombe* DNA polymerase α (Demagnez *et al.,* 1991); T.br.A, *Trypanosoma brucei* DNA polymerase α (Leegwater *et al.,* 1991); pAI2, *Ascobolus immersus* mitochondrion plasmid (Shu *et al.,* 1986); pCLKl, *Claviceps purpurea* linear plasmid (Oeser and Tudzynski, 1989); S1, *Zea mays S-1* mitochondrial DNA polymerase (Paillard *et al.,* 1985); pGKLl and pGKL2, *Kluyveromyces lactis* yeast linear killer plasmids 1 and 2 (Stark *et al.,* 1984; Tommasino *et al.,* 1988); PRD1, bacteriophage PRD1 (gram-negative hosts; Jung *et al.,* 1987); M2, *Bacillus subtilis* phage M2 (Matsumoto *et al.,* 1989); Phi29, *Bacillus subtillus* phage Phi29 (Yoshikawa and Ito, 1982); AD2, AD5, AD7, and AD12, human adenoviruses 2, 5, 7, and 12, respectively (Dekker and Ormondt, 1984; Engler *et al.,* 1983; Gingeras *et al.,* 1982; Shu *et al.,* 1986); E.co.II, *Escherichia coli* DNA polymerase II (Bonner *et al.,* 1990); H.s.D, *Homo sapiens* DNA polymerase δ (Yang *et al.,* 1992); BovD, bovine DNA polymerase δ (Ziang *et al.,* 1991); S.c.D, *Saccharomyces cerevisiae* DNA polymerase III (Boulet *et al.,* 1989); S.p.D, *Schizosaccharomyces pombe* DNA polymerase δ (Pignede *et al.,* 1991); P.falc.D, *Plasmodium falciparum* DNA polymerase δ (Ridley *et al.,* 1991); SAV, *Spodoptera* ascovirus; CHV, Chlorella virus (Grabherr *et al.,* 1992); EBV, Epstein–Barr virus (Baer *et al.,* 1984); HCMV, human cytomegalovirus (Kouzarides *et al.,* 1987); HSV1, herpes simplex virus type 1 (Gibbs *et al.,* 1985); VACV, vaccinia virus (Earl *et al.,* 1986); FPV, fowlpox virus (Binns *et al.,* 1987); CbEPV, *Choristoneura biennis* entomopoxvirus (Mustafa and Yuen, 1991); ASFV: African swine fever virus (Rodriguez *et al.,* 1993); T4, *E. coli* phage T4 (Spicer *et al.,* 1988); HzNPV, *Heliothis zea* nuclear polyhedrosis virus (GenBank Accession Number: GB_VI: NPVU11242); AcNPV, *Autographa californica* nuclear polyhedrosis virus (Tomalski *et al.,* 1988).

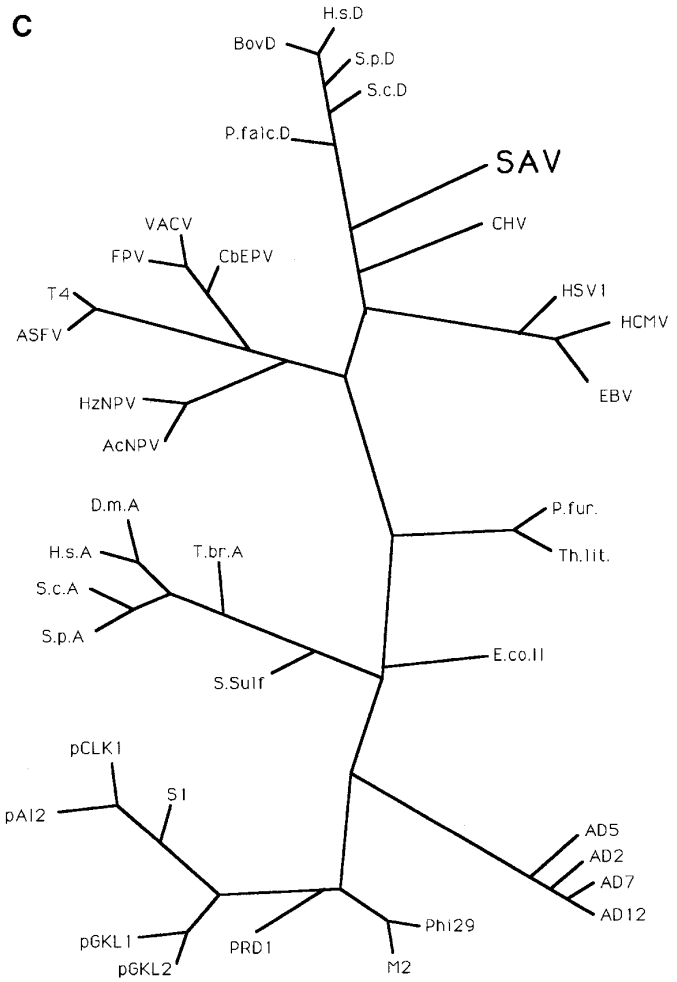

FIG. 6—*Continued*

AcMNPV share *S. frugiperda* as a host, it is not surprising that the SAV is able to function in this assay; we have not determined whether the SAV DNA polymerase can functionally replace the AcMNPV gene within the context of the virus genome and the infection process. The fact that the SAV DNA polymerase gene can substitute for the AcMNPV DNA polymerase in this assay provides strong genetic evidence that the ascovirus DNA gene we have cloned and sequenced encodes a functional DNA polymerase.

Construction of a series of unrooted phylogenetic trees based on complete peptide sequences or conserved sequence motifs from SAV and 37 other DNA polymerases establishes that SAV DNA polymerase is highly divergent from any of the previously characterized DNA polymerase classes. The basic tree we have derived by neighbor-joining analysis of the complete DNA polymerase sequences (Figs. 6A and 6C) agrees quite well with previously published phylogenetic DNA polymerase trees (Braithewaite and Ito, 1993; Uemori *et al.,* 1993) and has high branch integrity within the defined classes of DNA polymerases based on Bootstrap analysis (Fig. 6B). These branches can therefore be viewed with a high degree of confidence. The analysis places SAV DNA polymerase on a separate and distant branch at the base of the delta DNA polymerase class containing an organismally diverse set of DNA polymerases; the divergence times among the organisms represented in the delta DNA polymerase class (two mammals, two yeasts, and a protist) are large and the SAV polymerase is basal to this branch. SAV is also clearly not in the same virus family as chlorella virus, an algal virus, or any of the other DNA-containing virus families.

The interpretation of the tree in Fig. 6A and the divergent position of the SAV DNA polymerase gene is strongly supported by the results of trees generated using different algorithms and limiting the evaluation to the conserved motifs of DNA polymerases. These other five trees, although not as strong as the tree in Fig. 6A, also appear to place SAV DNA polymerase in its own class, often indicating an even weaker relationship between SAV DNA polymerase and the delta DNA polymerase class or the chorella virus.

There was one unexpected placement of DNA polymerases in the phylogenetic trees we constructed: the bacteriophage T4 and eukaryotic ASFV DNA polymerases were consistently placed in close proximity on a common branch (e.g., Fig. 6C). The T4 polymerase gene was selected for inclusion in our alignments as representative of the large DNA-containing phage polymerases while ASFV is currently considered to represent a unique (new) family of eukaryotic viruses. Our phylogenetic analysis of this polymerase gene is, to our knowledge, the first one performed for this virus and suggests an unexpected evolutionary relationship between ASFV and the T4 phage. Since viral DNA polymerase genes are usually essential genes, they are likely to have evolved closely with the virus and thus accurately reflect their evolutionary history. However, phylogenetic analyses of additional functionally similar genes of these two viruses is warranted before conclusions can be drawn concerning a common evolutionary origin.

The phylogenetic analyses of the ascovirus DNA polymerase gene sequence strongly support the hypothesis that ascoviruses are a novel virus family. Based on our analysis of this gene, the ascovirus genome appears to be distantly related to other viruses. Furthermore, the SAV DNA polymerase gene is distantly related to those of other insect viruses including the CbEPV entomopoxvirus and the baculovirus AcMNPV, which shares an insect host (*S. frugiperda*) with SAV. If the SAV DNA polymerase sequence and its flanking sequences are representative of the viral genome, SAV and probably other ascoviruses belong in a separate virus family, Ascoviridae.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service Grant AI23719 from the National Institute of Allergy and Infectious Diseases

and by the University of Georgia. We thank Russ Eldridge (FMC, Princeton, NJ) for pioneering the study of ascoviruses in the laboratory of L.K.M. and for helpful comments on the manuscript. We also thank Dr. Johnathan Arnold and Dr. Michael Arnold for advice on algorithm use, and Dr. Elena Prikhodko and Ms. Laura McMullan for laboratory advice and/or help.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.

Avery, S. W., Jouvenaz, D. P., Banks, W. A., and Anthony, D. W. (1977). Virus-like particles in a fire ant, *Solenopsis* sp. (Hymenoptera: Formicidae) from Brazil. *Fl. Entomol.* 60, 17–20.

Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrel, P. J., Gibson, T. J., Hatful, G., Hudson, G. S., Satchwell, S. C., Seguin, C., Tuffnell, P. S., and Barrell, B. G. (1984). DNA sequence and expression of the B95–8 Epstein–Barr virus genome. *Nature* 310, 207–211.

Bernad, A., Zaballos, A, Salas, M., and Blanco, L. (1987). Structural and functional relationships between procaryotic and eukaryotic DNA polymerases. *EMBO J.* 6, 4219–4225.

Bernad, A., Blanco, L., Lazaro, J. M., Martin , G., and Salas, M. (1989). A conserved 3′–5′ exonuclease active site in prokaryotic and eukaryotic DNA polymerases. *Cell* 59, 219–228.

Binns, M. M., Stenzler, L., Tomley, F. M., Campbell, J., and Boursnel, M. B. G. (1987). Identification by a random sequencing strategy of the fowlpoxvirus DNA polymerase gene, its nucleotide sequence and comparison with other viral DNA polymerases. *Nucleic Acids Res.* 15, 6563–6573.

Bonner, C. A., Hays, S., McEntee, K., and Goodman, M. F. (1990). DNA polymerase II is encoded by the DNA damage-inducible *dinA* gene of *Escherichia coli. Proc. Natl. Acad. Sci. USA* 87, 7663–7667.

Boulet, A., Simon, M., Faye, G., Bauer, G. A., and Burgers, P. M. J. (1989). Structure and function of the *Saccharomyces cerevisiae CDC2* gene encoding the large subunit of DNA polymerase III. *EMBO J.* 8, 1849–1854.

Braithewaite, D. K., and Ito, J. (1993). Compilation, alignment, and phylogenetic relationships of DNA polymerases. *Nucleic Acids Res.* 21, 787–802.

Browning, H. W., Federici, B. A., and Oatman, E. R. (1982). Occurrence of a disease caused by a Rickettsia-like organism in a larval population of the cabbage looper, *Trichoplusia ni,* in southern California. *Environ. Entomol.* 11, 550–554.

Carner, G. R. and Hudson, J. S. (1983). Histopathology of virus-like particles in *Heliothis* spp. *J. Invertebr. Path.* 41, 238–249.

Chaeychomsri, S., Ikeda, M., and Kobayashi, M. (1995). Nucleotide sequence and transcriptional analysis of the DNA polymerase gene of *Bombyx mori* nuclear polyhedrosis virus. *Virology* 206, 435–447.

Damagnez, V., Tillit, J., de Recondo, A., and Baldacci, G. (1991). The POL1 gene from the fission yeast, *Schizosaccharomyces pombe,* shows conserved amino acid blocks specific for eukaryotic DNA polymerases alpha. *Mol. Gen. Genet.* 226, 182–189.

Dekker, B. M. M., and van Ormondt, H. (1984). The nucleotide sequence of fragment *Hind*III-C of human adenovirus type 5 DNA. *Gene.* 27, 115–120.

Earl, P. L., Jones, E. V., and Moss, B. (1986). Homology between DNA polymerases of poxviruses, herpesviruses, and adenoviruses: Nucleotide sequence of the vaccinia virus DNA polymerase gene. *Proc. Natl. Acad. Sci. USA* 83, 3659–3663.

Engler, J. A., Hoppe, M. S., and van Bree, M. P. (1983). The nucleotide sequence of the genes encoded in early region 2b of human adenovirus type 7. *Gene* 21, 145–159.

Federici, B. A. (1982). A new type of insect pathogen in larvae of the clover cutworm, *Scotogramma trifolii. J. Invert. Pathol.* 40, 41–54.

Federici, B. A. (1983). Enveloped double stranded DNA insect virus with novel structure and cytopathology. *Proc. Natl. Acad. Sci.* 80, 7664–7668.

Federici, B. A. (1993). Viral pathobiology in relation to insect control. *In* "Parasites and Pathogens of Insects" (N. E. Beckage, S. N. Thompson, and B. A. Federici, Eds.), Vol 2, pp. 81–101. Academic Press, San Diego.

Federici, B. A. (1994). Ascoviruses. *In* "Encyclopedia of Virology" (R. G. Webster and A. Granoff, Eds.), Vol. 1, pp.58–63. Academic Press, London/San Diego.

Federici, B. A., and Govindarajan, R. (1990). Comparative histopathology of three ascovirus isolates in larval noctuids. *J. Invertebr. Pathol.* 56, 300–311.

Federici, B. A., Vlak, J. M., and Hamm, J. J. (1990). Comparative study of virion structure, protein composition and genomic DNA of three ascovirus isolates. *J. Gen. Virol.* 71, 1661–1668.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) Version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA.

Genetics Computer Group (1994). "Program Manual for the Wisconsin Package," Version 8, Genetics Computer Group, 575 Science Drive, Madison, WI, 53771.

Gibbs, J. S., Chiou, H. C., Hall, J. D., Mount, D. W., Retondo, M. J., Weller, S. J., and Coen, D. M. (1985). Sequence and mapping analyses of the herpes simplex virus DNA polymerase gene predict a C-terminal substrate binding domain. *Proc. Natl. Acad. Sci. USA* 82, 7969–7973.

Gingeras, T. R., Sciaky, D., Gelinas, R. E., Bing-Dong, J., Yen, C. E., Kelly, M. M., Bullock, P. A., Parsons, B. L., O'Neill, K. E., and Roberts, R. J. (1982). Nucleotide sequences from the adenovirus 2 genome. *J. Biol. Chem.* 257, 13475–13491.

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell Biol.* 2, 1044–1051.

Govindarajan, R., and Federici, B. A. (1990). Ascovirus infectivity and effects of infection on the growth and development of noctuid larvae. *J. Invertebr. Pathol.* 56, 291–299.

Grabherr, R., Strasser, P., and Van Etten, J. L. (1992). The DNA polymerase gene from chlorella viruses PBCV-1 and NY-2A contains an intron with nuclear splicing sequences. *Virology* 188, 721–731.

Hamm, J. J., Nordlund, D. A., and Marti, O. G. (1985). Effects of a nonoccluded virus of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on the development of a parasitoid, *Cotesia marginiventris* (Hymenoptera: Braconidae). *Environ. Entomol.* 14, 258–261.

Hamm, J. J., Pair, S. D., and Marti, O. G. (1986). Incidence and host range of a new ascovirus isolated from fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Fl. Entomol.* 69, 524–531.

Henikoff, S. (1987). Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol.* 155, 156.

Hirose, F., Yamaguchi, M., Nishida, Y., Masutani, M., Miyazawa, H., Hanaoka, F., and Matsukage, A. (1991). Structure and expression during development of *Drosophila melanogaster* gene for DNA polymerase alpha. *Nucleic Acids Res.* 19, 4991–4998.

Jung, G., Leavitt, M. C., Hsieh, J., and Ito, J. (1987). Bacteriophage PRD1 DNA polymerase: Evolution of DNA polymerases. *Proc. Natl. Acad. Sci. USA* 84, 8287–8291.

Kempken, F., Meinhardt, F., and Esser, K. (1989). *In organello* replication and viral affinity of linear, extrachromosomal DNA of the ascomycete *Ascolobus immersus. Mol. Gen. Gen.* 218, 523–530.

Kouzarides, T., Bankier, A. T., Satchwell, S. C., Weston, K., Tomlinson, P., and Barrel, B. G. (1987). Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. *J. Virol.* 61, 125–133.

Larder, B. A., Kemp, S. D., and Darby, G. (1987). Related functional domains in virus DNA polymerases. *EMBO J.* 6, 169–175.

Leegwater, P. A. J., Strating, M., Murphy, N. B., Kooy, R. F., van der Vliet, P. C., and Overdulve, J. P. (1991). The *Trypanosoma brucei* DNA polymerase alpha core subunit gene is developmentally regulated

and linked to a constitutively expressed open reading frame. *Nucleic Acids Res.* 19, 6441–6447.

Lu, A. L., and Miller, L. K. (1994). Identification of three late expression factor genes within the 33.8 to 43.4 map unit region of *Autograpa californica* nuclear polyhedrosis virus (AcMNPV). *J. Virol.* 68, 6710–6718.

Lu, A. L., and Miller, L. K. (1995). The roles of eighteen baculovirus late expression factor genes in transcription and DNA replication. *J. Virol.* 69, 975–982.

Manniatis, T., Fritsch, E. F., and Sambrook, J. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Marti, O. G., Hamm, J. J., and Styer, E. L. (1987). Discharge of hemolymph from the ventral eversible gland of ascovirus-infected fall armyworm larvae, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.* 49, 127–129.

Mathur, E. J., Adams, M. W. W., Callen, W. N., and Cline, J. M. (1991). The DNA polymerase gene from the hyperthermophilic marine archaebacterium, *Pyrococcus furiosis,* shows sequence homology with alpha-like DNA polymerases. *Nucleic Acids Res.* 19, 6952.

Matsumoto, K., Takano, Hiroyoshi, Kim, C. I., and Hirokawa, Hideo. (1989). Primary structure of bacteriophage M2 DNA polymerase: Conserved segments within protein-priming DNA polymerases and DNA polymerase I of *Escherichia coli. Gene* 84, 247–255.

Morrison, A., Bell, J. B., Kunkel, T. A., and Sugino, A. (1991). Eukaryotic DNA polymerase amino acid sequence required for 3′–5′ exonuclease activity. *Proc. Natl. Acad. Sci. USA* 88, 9473–9477.

Mustafa, A., and Yuen, L. (1991). Identification and sequenceing of the *Choristoneura biennis* entomopoxvirus DNA polymerase gene. *DNA Sequence* 2, 39–45.

Oeser, B., and Tudzynski, P. (1989). The linear mitochondrial plasmid pClKl of the phytopathogenic fungus *Claviceps purpurea* may code for a DNA polymerase and an RNA polymerase. *Mol. Gen. Genet.* 217, 132–140.

O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992). "Baculovirus Expression Vectors: A Laboratory Manual." Freeman, New York.

Paillard, M., Sederoff, R. R., and Levings, C. S. (1985). Nucleotide sequence of the S-1 mitochondrial DNA from the cytoplasm of maize. *EMBO J.* 4, 1125–1128.

Passarelli, A. L., and Miller L. K. (1993). Three baculovirus genes involved in late and very late gene expression: *ie-l, ien,* and *lef-2. J. Virol.* 67, 2149–2158.

Pearson, M. R., Bjornson, R., Pearson, G., and Rohrmann, G. (1992). The *Autographa californica* baculovirus genome: Evidence for multiple replication origins. *Science* 257, 1382–1384.

Pearson, W. R., and Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* 85, 2444–2448.

Perler, F. B., Comb, D. G., Jack, W. E., Moran, L. S., Qiang, B., Kucera, R. B., Benner, J., Slatsko, B. E., Nwankwo, D. O., Hempstead, S. K., Carlow, C. K. S., and Jannasch, H. (1992). Intervening sequences in an Archaea DNA polymerase gene. *Proc. Natl. Acad. Sci. USA* 89, 5577–5581.

Pignede, G., Bouvier, D., de Recondo, A., and Baldacci, G. (1991). Characterization of the POL3 gene product from *Schizosaccharomyces pombe* indicates inter-species conservation of the catalytic subunit of DNA polymerase delta. *J. Mol. Biol.* 222, 209–218.

Pisani, F. M., Martino, C. D., and Rossi, M. (1992). A DNA polymerase from the archaeon *Sulfolobus solfataricus* shows sequence similarity to family B DNA polymerases. *Nucleic Acids Res.* 20, 2711–2716.

Pizzagalli, A., Valsasnini, P., Plevani, P, and Lucchini, G. (1988). DNA polymerase I gene of *Saccharomyces cerevisiae:* Nucleotide sequence, mapping of temperature-sensitive mutation, and protein homology with other DNA polymerases. *Proc. Natl. Acad. Sci. USA* 85, 3772–3776.

Ridley, R. G., White, J. H., McAleese, S. M., Goman, M., Alano, P., deVries, E., and Kilbey, B. J. (1991). DNA polymerase delta: Gene sequences from *Plasmodium falciparum* indicate that this enzyme is more highly conserved than DNA polymerase alpha. *Nucleic Acids Res.* 19, 6731–6736.

Rodriguez, J. M., Yanez, R. J., Rodriguez, J. F., Vinuela, E., and Salas, M. L. (1993). The DNA polymerase-encoding gene of African swine fever virus: Sequence and transcriptional analysis. *Gene* 136, 103–110.

Shu, L., Hong, J. S., Wei, Y., and Engler, J. A. (1986). Nucleotide sequence of the genes encoded in early region 2b of human adenovirus 12. *Gene* 46, 187–195.

Spicer, E. K., Rush, J., Fung, C., Reha-Krantz, L. J., Karam, J. D., and Konigsberg, W. H. (1988). Primary structure of the T4 DNA polymerase. *J. Biol. Chem.* 263, 7478–7486.

Stark, M. J. R., Mileham, A. J., Romanos, M. A., and Boyd, A. (1984). Nucleotide sequence and transcription analysis of a linear DNA plasmid associated with the killer character of the yeast *Kluyveromyces lactis. Nucleic Acids Res.* 12, 6011–6030.

Thiem, S. M., and Miller, L. K. (1990). Differential gene expression mediated by late, very late, and hybrid baculovirus promotors. *Gene* 91, 87–94.

Todd, J. W., Passarelli, A. L., and Miller, L. K. (1995). Eighteen baculovirus genes, including *lef-11, p35, 39K,* and *p47* support late gene expression. *J. Virol.* 69, 968–974.

Tomalski, M., Wu, J., and Miller, L. K. (1988). The location, sequence, transcription, and regulation of a baculovirus DNA polymerase gene. *Virology* 167, 591–600.

Tommasino, M., Ricci, S., and Galeotti, C. L. (1988). Genome organization of the killer plasmid pGK12 from *Kluyveromyces lactis. Nucleic Acids Res.* 16, 5863–5878.

Uemori, T., Ishino, Y., Toh, H., Asada, K., and Kato, I. (1993). Organization and nucleotide sequence of the DNA polymerase gene from the archaeon *Pyrococcus furiosis. Nucleic Acids Res.* 21, 259–265.

Vaughn, J. L., Goodwin, R. H., Thompkins, G. L., and McCawley, P. (1977). The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *In Vitro* 13, 213–217.

Wang, T. S.-F., Wong, S. W., and Korn, D. (1989). Human DNA polymerase alpha: Predicted functional domains and relationships with viral DNA polymerases. *FASEB J.* 3, 14–21.

Wong, S. W., Wahl, A. F., Yuan, P., Arai, N., Pearson, B. E., Arai, K., Korn, D., Hunkapiller, M. W., and Wang, T. S.-F. (1988). Human DNA polymerase alpha gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. *EMBO J.* 7, 37–47.

Yang, C., Chang, L., Zhang, P., Hao, H., Zhu, L., Toomey, N. L., and Marietta Y. W. T. L. (1992). Molecular cloning of the cDNA for the catalytic subunit of human DNA polymerase delta. *Nucleic Acids Res.* 20, 735–745.

Yoshikawa, H., and Ito, J. (1982). Nucleotide sequence of the major early region of bacteriophage Phi 29. *Gene* 17, 323–335.

Ziang, J., Chung, D. W., Tan, C., Downey, K. M., Davie, E. W., and So, A. G. (1991). Primary structure of the catalytic subunit of calf thymus DNA polymerase delta: Sequence similarities with other DNA polymerases. *Biochemistry* 30, 11742–11750.